Reversal of Endocardial Endothelial Dysfunction By Folic Acid in Homocysteinemic Hypertensive Rats

Amanda Miller, Vibhas Mujumdar, Lena Palmer, John D. Bower, and Suresh C. Tyagi

The role of L- and D-isomers of homocysteine (Hcy) in vascular versus endocardial endothelial (EE) remodeling and function is not well understood. The hypothesis is that Hcy decreases EE cell density by activating matrix metalloproteinase (MMP) and by inducing left ventricular hypertrophy (LVH) in homocysteinemic hypertensive rats (HHR). And L- and D-isomers of Hcy have differential effects in vessel and myocardium. We used: 1) spontaneously hypertensive rats (SHR) in which endogenous total homocyst(e)ine (tHcy) levels are moderately high (18 μmol/L); 2) control age- and sex-matched normotensive Wistar rats (NWR) in which tHcy levels are normal (4 μmol/L); to create hyperhomocyst(e)inemia, 32 mg/day Hcy was administered for 12 weeks in 3) SHR (SHR-H), and in 4) NWR (NWR-H) rats; 5) endogenous tHcy levels were reduced (from 18 to 12 μmol/L) in SHR by folic acid administration (SHR-F). Plasma tHcy levels were measured by HPLC and spectrophometric methods. The MMP activity, measured by zymography, is increased by chronic Hcy administration, and folic acid treatment decreases MMP activity. The collagen and transforming growth factor-β1 (TGF-β1), measured by reverse transcriptase-polymerase chain reaction, are increased by Hcy. Folic acid treatment decreases collagen expression and increases TGF-β1. In vivo LV function was measured in anesthetized rats by a catheter in the left ventricle. The partial decrease in tHcy levels and no change in arterial pressure in SHR after folic acid administration, suggested that folic acid decreases one of the L- or D-isomer of Hcy, which is not responsible for an increase in arterial pressure, but may be responsible for myocardial dysfunction. The chronic Hcy administration decreases EE function in NWR and SHR. The treatment of folic acid in SHR improves LVH and EE function. Folic acid improves cardiac remodeling and EE function by decreasing one of the D- or L-isomer of Hcy and by decreasing MMP activity in HHR. These results may suggest a differential role of L- and D-isomers in vascular versus cardiac remodeling. Am J Hypertens 2002;15:157–163 © 2002 American Journal of Hypertension, Ltd.

Key Words: Gelatinase, growth factor, norepinephrine, electrocardiogram, left ventricular hypertrophy, cardiac ring, gene expression, functional genomic, hypertension, fibrosis, elastin, collagen, heart failure.

Hyperhomocyst(e)inemia is a causative agent for cardiovascular disease and is associated with systolic hypertension. The role of homocysteine (Hcy) in vascular endothelial dysfunction has been studied extensively; however, little attention is given to the role of Hcy in the capillary endocardial endothelium (EE). In an acute study we have demonstrated that Hcy impairs EE by decreasing the bioavailability of nitric oxide (NO). In a chronic study of 4 weeks hyperhomocysteinemia, Ungvari et al. have demonstrated that the reduced activity of NO in arterioles may contribute to the microvascular impairment by Hcy. However, the molecular mechanism of EE dysfunction by Hcy is unclear. In an ex vivo study, we have demonstrated that Hcy activates latent resident matrix metalloproteinase (MMP). In addition, in vivo inhibition of NO production increases MMP activity in other tissue. In culture conditions, inhibition of cytokine-induced nitric oxide synthase (NOS) reduced both expression and activity of MMP. In contrast, cytokine-inducible MMP in immortalized cells were not modified by NOS inhibition. The reasons for such diverse effects of NO on MMP are not clear. However, a differential regulation of MMP,
release and activation in vivo versus in vitro, may account for this discrepancy. The increase in NO production by L-arginine administration reduces left ventricular hypertrophy (LVH). However, the blood pressure (BP) response was unaffected in spontaneously hypertensive rats (SHR). Matthias et al. have demonstrated moderately high levels of total Hcy (tHcy) in SHR. This may imply that D- and L-isomers of Hcy may differentially regulate LVH versus BP. To test the hypothesis that D- and L-isomers of Hcy may have differential effect in impairing EE function by decreasing NO and increasing MMP activity, causing LVH and dysfunction, we used: 1) SHR in which endogenous tHcy levels are moderately high; 2) control age- and sex-matched normotensive Wistar rats (NWR) in which tHcy levels are normal; to create hyperhomocysteinemia, Hcy was administered in 3) SHR (SHR-H), and in 4) NWR (NWR-H) rats; 5) endogenous tHcy levels were reduced in SHR by folic acid administration, SHR-F. The results suggested that only one of the D- or L-isomer of Hcy impairs endocardial endothelium and not both.

Methods
Creation of Hyperhomocyst(e)inemia

There has been a recent international consensus that changes the terminology. According to new terminology, we use homocysteine as Hcy, and total homocysteine or homocyst(e)ine as tHcy. Because SHR resembles human hypertension and tHcy induces hypertension, and SHR have higher levels of tHcy as compared to age- and sex-matched NWR, we created a condition of chronic hyperhomocysteinemia by adding Hcy (0.67 mg/mL) to drinking water of normal and SHR. We did not administer methionine because methionine induces moderate homocyst(e)inemia and may effect overall protein synthesis. We created hyperhomocyst(e)inemia by directly administering Hcy as following: 1) SHR of 32 to 36 weeks, 325 to 350 g (Charles River Laboratories, Portland, ME) in which endogenous tHcy levels are moderately high; 2) control age- and sex-matched NWR in which tHcy levels are normal; to create hyperhomocysteinemia, Hcy was administered in 3) SHR (SHR-H), and in 4) NWR (NWR-H) rats; 5) endogenous tHcy levels were reduced in SHR by folic acid administration, SHR-F.

Cardiac Reactivity

Norepinephrine (NE) response was measured by infusing NE through a catheter in the jugular vein. At each injection of NE, the LV pressure was measured. The concentration of NE infused in the blood was determined based on the assumption that the rat has about 20 mL of blood volume. Five minutes after each dose of NE infusion, developed LV pressure (LVP) was recorded. A NE dose–response curve with LVP was constructed. The half-effective concentration (EC50) was determined by nonlinear least squares fit using:

\[ \text{LVP} = \left( \frac{A}{1 + \exp(B \text{Dose} - C)} \right) + D, \]

where A and D are constants and B is EC50.

Plasma tHcy

At the end of the measurements of functional parameters, 1 mL of blood was collected through a catheter in the right carotid. The plasma was separated and tHcy was separated by HPLC and measured by colorimetry.

After assessing LV function, the heart was arrested in diastole by injecting (intraperitoneally) 0.2 mL/100 g body weight of a 20% solution of KCl, rapidly excised, and placed in cold freshly prepared physiologic salt solution. To measure the ex vivo LV and right ventricular (RV) function, endocardial rings were prepared as described. The LV and RV wall thickness and diameters were measured with a digital micrometer.

EE Function

The “deli” shape LV rings were mounted in a tissue myobath. One of the two mounted wire was connected to a force transducer. The ring was stretched and brought to resting tension at which 20 mmol/L CaCl2 was added. At the maximum CaCl2 contraction, acetylcholine was added. Acetylcholine dose–response curves were generated. The percentage relaxation was calculated based on 100% con-
to 20 mmol/L CaCl₂. The EC₅₀ was determined using nonlinear least squares equation:

\[
\text{Percentage relaxation} = \left( A \left[ 1 + \exp \left( B \frac{\text{Dose}}{C} - D \right) \right] \right) + D,
\]

where A and D are constants and B is EC₅₀. The validity of the measurements regarding EE function using “deli” shape LV rings has been established by measuring rings response to cardiotoxic agents.³

### Preparation of Tissue Homogenates

The LV and RV tissue homogenates were prepared as described.¹⁷ A Bio-Rad dye binding assay was applied to estimate total protein. Total genomic DNA was isolated by DNAZol solution according to manufacturer’s recommendation. Isolated DNA was measured at 260 nm using an optical density of 1 for 50 μg/mL of DNA.

### Zymography

To determine total MMP activity in the left ventricle of rats from these five study groups, a gelatin-zymography was performed. SDS-PAGE containing 1% gelatin was used as impregnated substrate for MMP.¹⁷ The gels were stained by Coomassie blue and lytic bands were scanned by a Bio-Rad GS-700 densitometer (Richmond, CA).

### Actin Western Blot

To determine whether total proteins loaded onto the gel were identical, α-actin Western blots were performed, using anti-actin antibody (Sigma). The alkaline phosphatase conjugated secondary antibody was used as detection system. The bands on the blots were scanned.

### RT-PCR Analysis of Collagen and Transforming Growth Factor-α₁

Total RNA was isolated using RNAzol solution according to manufacturer’s instruction. The following sets of primers of respective genes were used: rat transforming growth factor-β₁ (TGF-β₁): upstream 5’-CTTCAGCTCCACA-GAGAAAGACTGC-3’ (1267 to 1291 bp); downstream 5’-CACGATCATAGTTGGACACTGCTCC-3’ (1564 to 1540 bp); rat collagen I: upstream 5’-GGACACAAATGG-ATTGCAAGG-3’; downstream 5’-TAAACCTGCTCC-ACTCTGG-3’; rat glyceraldehyde 3-P dehydrogenase (G3PDH): upstream 5’-TGAAGGTCGGTGTCACG-GATTTGGC-3’ (35 to 60 bp); downstream 5’-CATGTA-GCCATGGATGTCACCCAC-3’ (1017 to 994). RT-PCR was performed as described.¹⁸ The bands for respective PCR products were scanned and normalized with control G3PDH as a housekeeping gene.

### Statistical Analysis

Data was presented as mean ± SEM. Because the aim of this study was to compare the effect of Hcy on normoten- sive and hypertensive, separately, as well as the effect of folic acid on homocysteinemic hypertensive rats, SHR (in which endogenous tHcy is high). Therefore, the data in

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**Table 1.** Plasma tHcy, mean arterial pressure (MAP), and cardiac structural and functional parameters in NWR, NWR-H, SHR, SHR-H, and SHR-F

<table>
<thead>
<tr>
<th></th>
<th>NWR</th>
<th>NWR-H</th>
<th>SHR</th>
<th>SHR-H</th>
<th>SHR-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>tHcy (μmol/L)</td>
<td>3.7 ± 0.3</td>
<td>32.4 ± 0.7*</td>
<td>18.1 ± 0.5</td>
<td>28.4 ± 1.8†</td>
<td>12.5 ± 0.7</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>95 ± 5</td>
<td>126 ± 8*</td>
<td>157 ± 10</td>
<td>188 ± 5†</td>
<td>165 ± 12</td>
</tr>
<tr>
<td>LVP (mm Hg)</td>
<td>75 ± 25</td>
<td>122 ± 15*</td>
<td>110 ± 45</td>
<td>153 ± 3</td>
<td>124 ± 53</td>
</tr>
<tr>
<td>-dP/dt (mmHg/sec)</td>
<td>4399</td>
<td>2156 ± 189</td>
<td>3135 ± 1261</td>
<td>1568 ± 588</td>
<td>4449 ± 2816</td>
</tr>
<tr>
<td>LV wall (mm)</td>
<td>4 ± 1</td>
<td>3 ± 2</td>
<td>4.5 ± 1.5</td>
<td>2.5 ± 0.5</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>RV wall (mm)</td>
<td>2 ± 1</td>
<td>1.5 ± 0.5</td>
<td>3 ± 1</td>
<td>1.75 ± 0.25</td>
<td>0.96 ± 0.12</td>
</tr>
<tr>
<td>LV diameter (mm)</td>
<td>8 ± 1</td>
<td>10.5 ± 0.5</td>
<td>9 ± 1</td>
<td>9.5 ± 1.5</td>
<td>9.7 ± 0.5</td>
</tr>
<tr>
<td>RV diameter (mm)</td>
<td>7 ± 1</td>
<td>10 ± 1</td>
<td>8 ± 1</td>
<td>8.75 ± 1.25</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td>LV DNA (ng/mg)</td>
<td>605 ± 15</td>
<td>424 ± 19*</td>
<td>480 ± 40</td>
<td>630 ± 18</td>
<td>487 ± 45</td>
</tr>
<tr>
<td>RV DNA (ng/mg)</td>
<td>465 ± 12</td>
<td>475 ± 22</td>
<td>340 ± 10</td>
<td>370 ± 28</td>
<td>335 ± 25</td>
</tr>
<tr>
<td>LV protein (mg/mg)</td>
<td>0.35 ± 0.09</td>
<td>0.36 ± 0.11*</td>
<td>0.32 ± 0.06</td>
<td>0.46 ± 0.14</td>
<td>0.24 ± 0.03†</td>
</tr>
<tr>
<td>RV protein (mg/mg)</td>
<td>0.23 ± 0.16</td>
<td>0.27 ± 0.12</td>
<td>0.25 ± 0.13</td>
<td>0.35 ± 0.10</td>
<td>0.18 ± 0.06‡</td>
</tr>
<tr>
<td>LV collagen (μg/mg)</td>
<td>0.59 ± 0.08</td>
<td>0.86 ± 0.12*</td>
<td>0.59 ± 0.31</td>
<td>0.76 ± 0.11†</td>
<td>0.61 ± 0.12</td>
</tr>
<tr>
<td>RV collagen (μg/mg)</td>
<td>1.0 ± 0.2</td>
<td>2.54 ± 0.08</td>
<td>1.98 ± 0.54</td>
<td>2.49 ± 0.33</td>
<td>2.15 ± 0.18</td>
</tr>
<tr>
<td>LV elastin (μg/mg)</td>
<td>0.03 ± 0.02</td>
<td>0.0400 ± 0.001</td>
<td>0.01 ± 0.001</td>
<td>0.008 ± 0.001</td>
<td>0.01 ± 0.005</td>
</tr>
<tr>
<td>RV elastin (μg/mg)</td>
<td>0.08 ± 0.01</td>
<td>0.050 ± 0.007</td>
<td>0.10 ± 0.001</td>
<td>0.07 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
</tbody>
</table>

NWR = normotensive Wistar rats; NWR-H = normotensive Wistar rats treated with Hcy; SHR = spontaneously hypertensive rats; SHR-H = spontaneously hypertensive rats treated with Hcy; SHR-F = spontaneously hypertensive rats with folic acid; tHcy = total homocyst(e)ine; LVP = left ventricular pressure; EDP = end-diastolic pressure; LV = left ventricle; RV = right ventricle.

Each rat was anesthetized with intraperitoneal Inactin (100 mg/kg). After hemodynamic measurements, tissue from the LV and the RV was isolated. The mean ± SEM was reported.

* Compared NWR-H with NWR; † compared SHR-H with SHR; and ‡ compared SHR-F with SHR. *, †, ‡ P < .04, considered significant. The mg represents mg of tissue.
Table 1 and Figs. were compared as follows: NWR with NWR-H; SHR with SHR-H; and SHR with SHR-F. The significance of the data was tested by Student unpaired t test. The highest P value among the groups was reported when compared NWR-H with NWR; compared SHR-H with SHR; and compared SHR-F with SHR.

Results

Hcy Increases LV MMP Activity

Zymographic analysis revealed constitutive expression of MMP at 72 kD in all groups. The activity at 92 and 66 kD was specifically increased in Hcy-treated NWR and SHR. The treatment of folic acid in SHR decreased the MMP activity at 72 and 66 kD and had no effect at 92 kD (Fig. 1).

Cardiac Fibrosis and Hcy

Homocysteine increased collagen and TGF-β1 mRNA in NWR and SHR (Fig. 2). Folic acid administration decreased collagen expression, and increased TGF-β1 mRNA in homocysteinemic hypertensive rats, SHR.

LV Hypertrophy

Protein/DNA ratio was increased in the LV of NWR-H rats as compared to NWR (Fig. 3), suggesting that Hcy induced LV hypertrophy in normal rats. Homocysteine also induces LVH and RVH and the treatment of folic acid regresses the LVH and RVH in SHR.

Hcy, Folic Acid, and EE Function

The levels of endogenous tHcy were elevated in SHR as compared to NWR (Table 1). The response to acetylcho-
line was suppressed in SHR as compared to NWR. Chronic Hcy administration impaired EE function in both NWR and SHR. The treatment of SHR with folic acid improves the EC\textsubscript{50} of acetylcholine response (Fig. 4C), however, the maximum dose–response did not improve when compared to NWR (Fig. 4B).

**Hcy Impairs Cardiac Reactivity**

Dose–response curves suggested maximum LV pressure of 118 to 128 mm Hg in NWR and NWR-H; and 145 to 160 mm Hg in SHR, SHR-H, and SHR-F (Fig. 5B). The EC\textsubscript{50} of norepinephrine response in LVP was decreased in NWR after Hcy administration, suggesting that NE has significant effect on LV reactivity in NWR and not as much in SHR (Fig. 5C). The folic acid treatment has no effect on LV reactivity to NE in homocysteinemic hypertensive rats, SHR.

**LV Parameters**

The LV function, end-diastolic pressure (EDP), cardiac output, and −dP/dt/MAP were measured in Hcy-treated rats. The EDP was increased and −dP/dt/MAP was reduced in SHR-F, SHR-H, and SHR-H compared to NWR. The EDP was decreased in SHR after folic acid administration, suggesting a role of folic acid in improvement of cardiac function in homocysteinemic hypertensive rats (Fig. 6).

**Hcy Impairs Cardiac Conductance**

In homocysteinemic hypertensive rats, SHR, depression in S-T segment was observed, and the duration between P and Q was shortened after Hcy administration. The treatment of folic acid reverses this shortening in P and Q duration (Fig. 7C–E). There was no change in S-T segment in NWR with or without Hcy administration. However, the duration of QRS complex (cardiac depolarization) was increased in NWR and SHR treated with Hcy. The treatment with folic acid reduces QRS duration in SHR (Fig. 7F).
HOMOCYST(E)INE INDUCES CARDIAC HYPERTROPHY

FIG. 7. Electrocardiogram of NWR, NWR-H, SHR, SHR-H, and SHR-F. Rats were anesthetized with intraperitoneal Inactin (100 mg/kg). The three electrode (left and right legs and right arm) electrocardiogram was recorded. The QRS duration (in milliseconds) was estimated by a computerized Micro-Med Software. 

Discussion

The mechanism by which Hcy impairs EE function is, in part, associated with activation of MMP and induction of LVH, leading to impaired active and passive LV function. Other investigators have shown that the treatment of folic acid improves endothelial function by decreasing the levels of tHcy. Here we demonstrate that folic acid ameliorates Hcy-mediated EE dysfunction, and decreases LVH by decreasing MMP activity in homocysteinemic hypertensive rats. Although SHR has higher basal levels of plasma tHcy than NWR, the treatment with folic acid demonstrated moderate reduction in tHcy levels, but had no effect on arterial pressure (Table 1). This may indicate, in part, that SHR probably has the mixture of both D- and L-enantiomers of Hcy, and folic acid was unable to reduce one of the isomers of Hcy. The increase in arterial pressure by Hcy may be attributed to the remaining isomer of Hcy. This may suggest that folic acid reduces the isomer that does not contribute to the increase in arterial pressure. Alternatively, because folic acid reduces free plasma Hcy, independent of changes in protein-bound Hcy, it is also possible that protein-bound Hcy may be associated with arterial dysfunction and hypertension.

Homocysteine treatment increases MMP at 66 and 92 kD in both NWR and SHR. The folic acid treatment reduces, in part, the MMP activity at 66 and 72 kD (Fig. 1). Because 66 kD MMP is proteolytically converted from 72 kD, it is conceivable that other serine proteinase, such as plasminogen activators, are activated by Hcy. In fact studies have demonstrated that a serine elastase is activated by Hcy in the vessel wall. These results may point to the mechanism by which Hcy induces cardiac dilatation, and systolic dysfunction.

Homocysteine induces collagen expression in NWR as well as in SHR (Fig. 2). The TGF-β1 is one of the instigating factors causing fibrosis. The results suggest that Hcy induces TGF-β and may, in part, be responsible for an autocrine/paracrine link between cardiac fibrosis in hyperhomocysteinemia. Treatment with folic acid, however, reduces the expression of collagen, but increases TGF-β in homocysteinemic hypertensive rats, SHR. These results may suggest two independent roles of folic acid: 1) in the induction of TGF-β1 and 2) in the decrease of collagen expression. This may elicit a differential role of D- or L-isomer in regulation of TGF-β1 and collagen expression.

The treatment of Hcy instigates LV hypertrophy in NWR and SHR (Fig. 3). The Hcy-induced hypertrophy was higher in RV than LV and the folic acid treatment reduced partially the Hcy-associated hypertrophy in SHR. Again, this may suggest that the Hcy isomer, which is detrimental to LV, is decreased by folic acid in SHR. Also, a differential role of D- or L-isomer in RV versus LV of SHR may be implicated. It is of great interest to determine whether Hcy-induced LV hypertrophy in NWR is repressed by folic acid. These studies are in progress.

The response to acetylcholine was impaired in NWR and SHR treated with Hcy (Fig. 4). The folic acid treatment improves, in part, acetylcholine response in SHR. We may speculate that the treatment of folic acid reduces the isomer of Hcy, which is detrimental to endocardial endothelium.

The dose–response curve of NE demonstrated increased receptor sensitivity in NWR-H and SHR as compared to NWR. There was no difference in the receptor sensitivity in SHR or SHR-H (Fig. 5). The results may suggest differential regulation of cardiotonic receptors by Hcy in SHR than NWR. The LV active and passive diastolic function was decreased in both NWR and SHR after Hcy administration. The treatment of folic acid improved LV function (Fig. 6).

Cardiac depolarization was slowed by Hcy in NWR and SHR. The treatment of folic acid demonstrated decreased QRS duration in SHR (Fig. 7). The discontinuity in ECG and elongation of cardiac depolarization in NWR-H, SHR, and SHR-H may suggest an association of increased cardiac fibrosis (ie, collagen) by Hcy.

Perspective

The treatment with folic acid demonstrated moderate reduction in tHcy levels, but had no effect on BP (Table 1). These results may suggest that L- and D-isomers of Hcy have different effects, one affecting the vessel and other affecting the heart. To annex the effect of D- from DL-isomer in vessel versus myocardium, it is essential to...
prepare pure D-isomer of Hcy by reducing D-homocystine (Sigma). This is the first report that attempts to separate the effects of D- and L-isomers of Hcy in cardiac versus vascular remodeling. The possibility exists that SHR is homozygous/heterozygous in cystathione β synthase (CBS) activity. It is of great interest to determine whether folic acid reduces cardiac hypertrophy in normotensive rats treated with Hcy. Also, it is important to determine whether the early treatment of SHR at 2 to 4 weeks of age with folic acid will reduce LVH and hypertension. These studies are in progress.

References